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BASIC INVESTIGATION

Modulation of expression of P16 and Her2 in rat breast tissues of mammary hyperplasia model by external use of Rupifang Extract

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Abstract

OBJECTIVE: To observe the effect of Rupifang Extract in external use on expression of proto-oncogenes her2 and tumor suppression genes p16 in rat breast tissues of mammary hyperplasia model. To explore the mechanisms of Rupifang Extract in external use for preventing and treating mammary hyperplasia.

METHODS: Thirty virginal female Wistar rats were randomized into 5 groups, 6 in each, A: blank control group; B: model group; C: the low dose group of Rupifang; D: the middle dose group of Rupifang; and E: The high dose group of Rupifang. The mammary hyperplasia rat models were produced by injecting estradiol benzoate and progesterone and irritating by tail nipping. Drug intervention was also launched during the model formation. After 30 days, the expression of her2 and p16 in breast tissues of rats in each group were detected by the SP immunohistochemical method.

RESULTS: Compared with Blank control group, the expression of her2 in breast tissues in Model group was higher, and the expression of p16 was lower ($P<0.05$ or $P<0.01$). After intervention with Rupifang Extract, compared with Model group, the expression of her2 in breast tissues in Rupifang groups was lower, and the expression of p16 higher ($P<0.05$ or $P<0.01$).

CONCLUSION: The mechanisms of Rupifang Extract in external application for preventing and treating mammary hyperplasia may be reducing the expression of proto-oncogenes her2 and increasing the expression of tumor suppression genes p16.

Key words: Fibrocystic Breast Disease; Receptor, Epidermal Growth Factor; Rupifang extract

INTRODUCTION

Modern medicine considers that the occurrence of mammary hyperplasia is related to endocrine disturbances. So far there is no special drug to treat it. Hormone preparation is commonly used to relieve symptoms temporarily. However, its side effects are serious and the curative effect in long-time using is dubious. Focal surgical treatment is hardly accepted by patients, and it is easy to recur.

Traditional Chinese Medicine (TCM) treatment of mammary hyperplasia has gained quite great progress in recent years, in which, external therapy is unique and good in clinical efficacy. Our research group has obtained a significant effect in the treatment of mammary hyperplasia by Rupifang prescriptions since 2003.

This research was to observe the effect of Rupifang Extract in external application on the pathomorphology and expression of her2 and p16 in rat breast tissues of mammary hyperplasia model, and explore the mechanisms of Rupifang in preventing and treating mammary hyperplasia.

MATERIALS AND METHODS

Animals

Thirty SPF level virginal Wistar rats [weight (200±20)g] purchased from the Animal Experiment Center of Southern Medical University, Guangdong Province (Quality Permit No. SCXK Guangdong 2006-0015).

Reagents and drugs

Rabbit-Anti-Human her2 and Rabbit-Anti-Human p16 SP immunohistochemical kit (Fuzhou Maixin Biological Technology Development Co., Ltd.); DAB chromogenic reagents (Beijing Zhongshan Gold Bridge Biotechnology Co., Ltd.); Estradiol Benzoate Injection: 1ml:1mg (Tianjin Jinyao Amino Acid Co., Ltd.); Progesterone injection: 1 ml∶20 mg (Tianjin Jinyao Amino Acid Co., Ltd.).

Rupifang: composed of Rose (Flos Rosae Rugosae), Dingxiang (Flos Caryophylli), Dahuang (Radix et Rhizoma Rhei), Tougucao (Herba Speranskiae Tuberculatae), Yanhusuo (Rhizoma Corydalis), Wangbuliuxing (Semen Vaccariae). The proportion of each herb was 1∶1. The herbs were decocted for 1 hour, filtrated, and the decoction was condensed into extract. Each milliliter extract (about 2 g) contained about 2 g crude drugs.

Groups and treatment

Thirty rats were randomized into 5 groups, 6 in each. A. Blank control group; B. Model group; C. The low dose group of Rupifang; D. The middle dose group of Rupifang; and E. The high dose group of Rupifang. After feeding for 1 week, the rats had regular breast depilation on each pair of breasts by hair-removal cream. Modeling and drug intervention were started. From the 2nd day of the experiment to the 30th days, the rats in group A were injected physical saline (0.5 mL/kg per day) into the muscle of medial side of hind leg for 25 day and physical saline (0.2 mL/kg per day) for 5 days. The rats in group B, C, D and E were irritated by tail nipping 0.5 h/day, 30 days. The rats in group B were injected Estradiol Benzoate Injection (0.5 mL/kg per day) into the muscle of medial side of hind leg for 25 days and injected Progesterone (4 mg/kg per day) for 5 days. The rats in group C were treated in the same way for modeling as group B, at the same time, treated with Rupifang Extract (0.2 g) for external application in the region of breasts, once a d, and lmin massage for each breast was given, 30 days. The rats in group D were treated in the same way for modeling as group B, at the same time, treated with Rupifang Extract (0.4 g) for external application in the region of breasts, once a day, and lmin massage for each breast was done, 30 days. The rats in group E were treated in the same way for modeling as group B, at the same time, treated with Rupifang Extract (0.8 g) for external application in the region of breasts, once a day, and lmin massage for each breast was carried out, 30 days.[1] All rats were fed whole value grain feedstuff and tap-water ad libitum. In order to apply Rupifang Extract more effectively, the rats had breasts depilation by hair-removal cream regularly.

Specimen collection and index measurement

On the 30th day, the rats were anesthetized with 20% urethane (1.0 g/kg) through intraperitoneal injection. The 2nd and 3rd pairs of breasts were removed, fixed with 10% formaldehyde, and dehydrated by using gradient ethanol, embedded with paraffin, sliced (4 μm), and stained with HE. Referring to the standard mentioned in literature,[2] the observation with microscope on the hyperplasia of breast tissues was performed. Referring to the judgment standard mentioned in literature,[3,4] the expressions of her2 and p16 in breast tissues in each group were detected by the SP immunohistochemical method.

Statistical analysis

The experimental data was analyzed by SPSS 11.5. Results of measurement were expressed by $\bar{x} \pm s$. Differences between groups were assessed by one-way ANOVA. Paired comparisons were carried out by *t*-test. Results of ranked data were analyzed by non-parametric rank-sum test.

RESULTS

Effect of Rupifang Extract on histomorphology in rat breast tissues

There was no hyperplasia in breast tissues in Group A. Severe hyperplasia in breast tissues was found in Group B, there was hyperplasia in most lobules, significant expansion of most acini and ducts, increase of epithelioglandular layers, and a large amount of secretion in the acini and ducts. Moderate and severe hyperplasia was in Group C. Moderate hyperplasia was in Group D, there was hyperplasia in most lobules, significant expansion of some acini and ducts, increase of some epithelioglandular layers, and secretion in the acini and ducts. There existed the most obvious improvement of pathomorphological changes in Group E, it's only of mild and moderate hyperplasia in a few acini, no expansion of ducts, and a little secretion in some acini and ducts. (Figure 1). Statistical analysis showed no hyperplasia of breast tissues in Group A, compared with which, the significant difference was presented in Group B (P<0.01), meaning that exogenous hormone in combination with emotional stimulus could produce mammary hyperplasia animal models well. With the external application of Rupifang Extract, the hyperplasia of breast tissues was reduced (P<0.01), and the middle- and high-dose groups were better in effect than the low-dose group (P<0.01) (Table 1).

Effect of Rupifang Extract on expression of her2 in rat breast tissues

The expression of her2 in breast tissues of rats in Group A was negative. Compared with Group A, the positive expression of her2 in breast tissues of rats in Group B was obviously increased (P<0.01 or P<0.05). The external application of Rupifang Extract made the expression of her2 in breast tissues in the model groups significantly reduced (P<0.01), there existed significant differences among Rupifang groups (P<0.01), the expression of her2 was lower in the middle- and high-dose groups than in the low-dose group of Rupifang (P<0.01), and there was no difference between the middle- and high-dose groups (Table 2, Figure2).

Effect of Rupifang Extract on expression of p16 in rat breast tissues

Compared with Group A, the positive expression of p16 in breast tissues in Group B was obviously reduced (P<0.01). After intervention with Rupifang Extract, compared with Group B, the positive expression of p16 in Group C, D, and E was increased. There was significant difference (P<0.01) between Group D and B (P<0.01) (Table 3, Figure 3).

DISCUSSION

Rupifang is made of Chinese herbal medicines which are able to disperse liver stagnation, promote *Qi* circulation, activate blood, and remove stasis. This prescription, made into Chinese herbal plaster,[5] is used to treat mammary hyperplasia of phlegm accumulated with blood stasis. In the clinical trial, 50 mammary hyperplasia patients were treated effectively owing to its functions to improve local blood circulation, restrain hyperplasia of mammary gland, promote the absorption of fibrous tissues, and relieve pain.

Modern researches demonstrated that the chronic emotional stimulation could cause changes of function of immune system in rats, thus some cytokines were released to activate the hypothalamic-pituitary-adrenocortica (HPA) axis, then the released corticosterone (CORT) made the secretion of hypothalamic-pituitary-ovarian (HPO) axis disordered. Consequently, a series of changes in function of immune system appeared, leading to mammary hyperplasia and breast cancer eventually.[6]

Mammary hyperplasia is a hormone dependent disease, its occurrence is related to out-of-proportion of estrogen and progestogen. Estrogen in majority of woman is absolutely or relatively high, while progestogen low. Estrogen can stimulate the mammary epithelial and mesenchymal cells to be hyperplastic. Progestogen can resist estrogen partly and improve the proliferation of acini. The general method to establish animal models of the mammary hyperplasia is to use estrogen and progestogen in combination.[7]

In this research the mammary hyperplasia animal models were established by intramuscular injection with exogenous estradiol and progesterone combined with emotion stimulus. Histology of breast tissues in each model group showed severe hyperplasia of breast tissues: hyperplasia of most breast lobules, remarkable expansion of most acini and ducts, obvious increase of epithelial layers, and a large amount of secretion in the acini and ducts. It means that exogenous hormones (combined with emotion stimulus) can produce the animal model of mammary hyperplasia. After intervention with Rupifang Extract, the hyperplasia of breast tissues reduced, suggesting that it's effective to relieve mammary hyperplasia.

Her2 is an oncogene that is most closely related to breast cancer. It shows her2 gene amplification and over-expression of its gene product. So her2 antibody can be used to display on frozen or paraffin sections. The strong positive expression of her2 can be regarded as an index to identify early breast cancer. The amplification and over-expression of her2 are closely related to metastasis and recurrence of cancer and survival time of patient.

Kang's study[8] shows that the detection of her2 has a certain practical reference value on evaluating breast duct epithelial atypical hyperplasia and its canceration potential. The research of Cai BY[9] suggests that the positive expression of her2 occurring in atypical hyperplasia period plays an important role in the process from

Table 1 Histomorphological changes

Group	Number of rats	Number of breast	Normal (–)	Slight hyperplasia (+)	Moderate hyperplasia (++)	Severe Hyperplasia (+++)
A	6	24	24	0	0	0
B[a]	6	24	0	0	0	24
C[ab]	6	24	0	4	12	8
D[abc]	6	24	0	8	16	0
E[abc]	6	24	0	12	12	0

Notes: A: blank control group; B: model group; C: the low dose group of Rupifang; D: the middle dose group of Rupifang; E: the high dose group of Rupifang; Compared with group A, [a]P<0.01; Compared with group B, [b]P<0.01; Compared with group C, [c]P<0.01.

Table 2 Expression of her2 in rat breast tissues

Group	n	Number of breasts	(–)	(+)	Positive rate (%)
A	6	24	24	0	0
B[a]	6	24	0	24	100
C[a]	6	24	0	24	100
D[acd]	6	24	16	8	33
E[bcd]	6	24	20	4	17

Notes: A: blank control group; B: model group; C: the low dose group of Rupifang; D: the middle dose group of Rupifang; E: the high dose group of Rupifang; Compared with group A, [a]P<0.01, [b]P<0.05; Compared with group B, [c]P<0.01; Compared with group C, [d]P<0.01.

Table 3 Expression of p16 in rat breast tissues

Group	n	Number of breasts	(–)	(+)	(++)	(+++)
A	6	24	0	0	0	24
B[a]	6	24	0	0	8	16
C[b]	6	24	0	0	4	20
D[c]	6	24	0	0	0	24
E[b]	6	24	0	0	4	20

Notes: A: blank control group; B: model group; C: the low dose group of Rupifang; D: the middle dose group of Rupifang; E: the high dose group of Rupifang; Compared with group A, [a]P<0.01, [b]P<0.05; Compared with group B, [c]P<0.01.

mammary hyperplasia to breast cancer.

The decrease or the deletion and inactivation of tumor suppression gene p16 is closely related to the degree of mammary hyperplasia and the occurrence and prognosis of breast cancer. Along with the severity of breast duct epithelial atypical hyperplasia, the positive expression of p16 is getting decreased.[10,11]

In this research, after the mammary hyperplasia animal model was established by exogenous hormones (estradiol benzoate and progesterone) combined with tail pinching, the expression of her2 in breast tissues was higher and the expression of p16 was lower in the model group compared with the blank control group (P< 0.05 or P<0.01). Intervention with application of Rupifang Extract makes the expression of her2 in breast tissues reduced significantly (P<0.01) and the expression of p16 increased. It means that Rupifang can suppress or reverse the hyperplasia, uncontrolled proliferation, and malignant transformation of breast epithelium, being one of mechanisms of Rupifang for preventing and treating mammary hyperplasia.

Mammary hyperplasia, especially atypical hyperplasia, is closely related to breast cancer, the incidence of mammary hyperplasia is on the rise nowadays. It is an important task to find more convenient and effective drugs with few side effects and to explore the mechanisms of these drugs for blocking its development to cancer.

This research is that to establish the mammary hyperplasia animal model by exogenous hormones combined with tail pinching and apply Rupifang Extract for intervention, and a good effect on the prevention and treatment for mammary hyperplasia animal model is achieved. Its mechanisms may be that the expression of proto-oncogene her2 is reduced and the expression of tumor suppression gene p16 increased. This result provides theoretical basis for clinical application of Rupifang.

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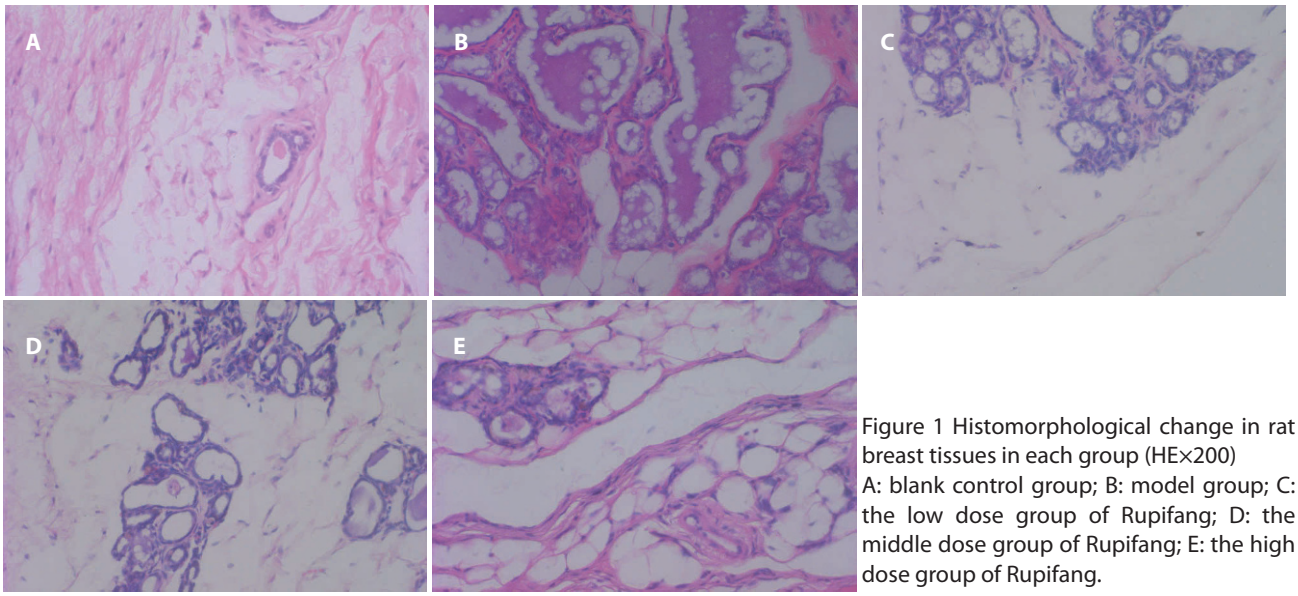

Figure 1 Histomorphological change in rat breast tissues in each group (HE×200)
A: blank control group; B: model group; C: the low dose group of Rupifang; D: the middle dose group of Rupifang; E: the high dose group of Rupifang.

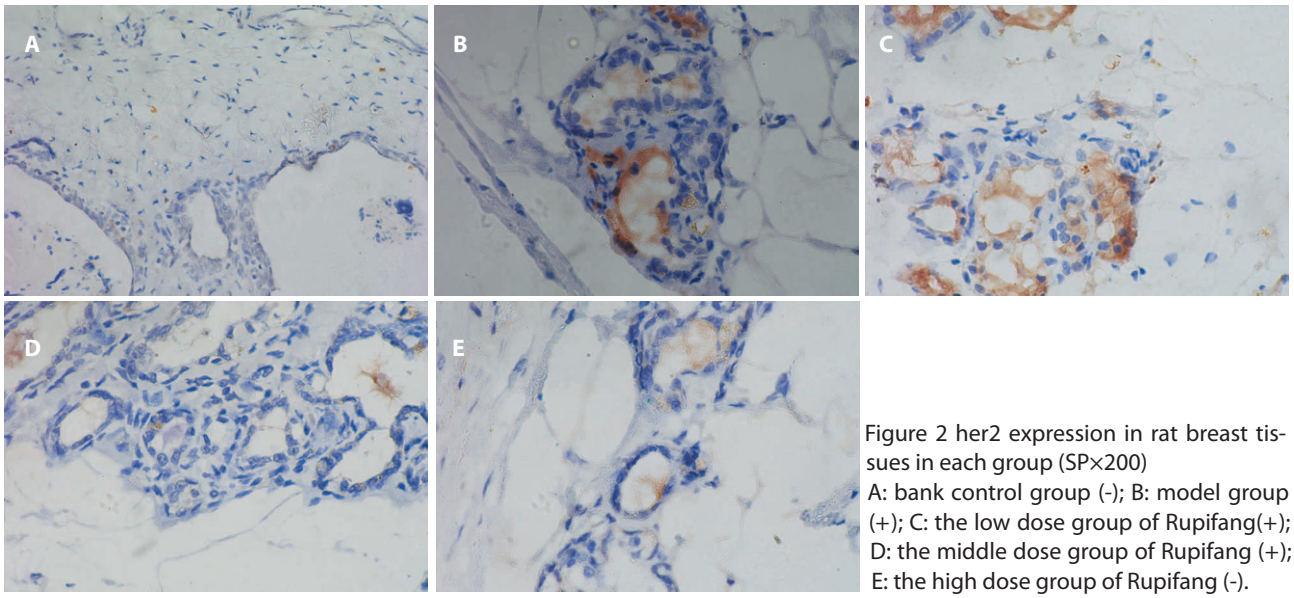

Figure 2 her2 expression in rat breast tissues in each group (SP×200)
A: bank control group (-); B: model group (+); C: the low dose group of Rupifang(+); D: the middle dose group of Rupifang (+); E: the high dose group of Rupifang (-).

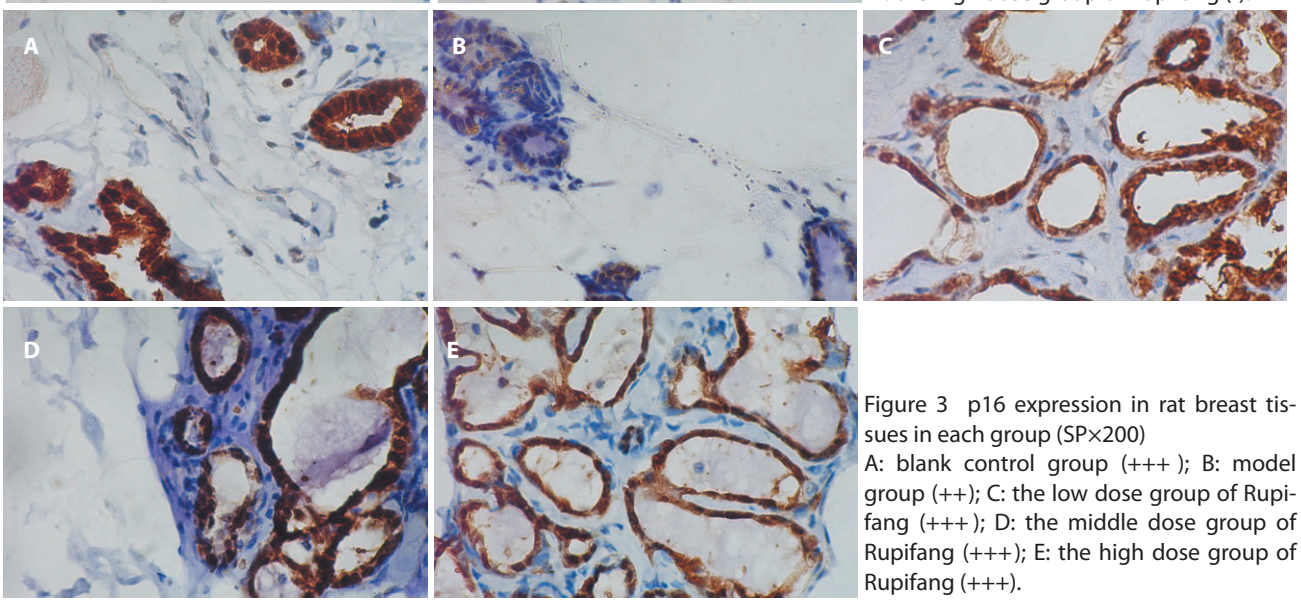

Figure 3 p16 expression in rat breast tissues in each group (SP×200)
A: blank control group (+++); B: model group (++); C: the low dose group of Rupifang (+++); D: the middle dose group of Rupifang (+++); E: the high dose group of Rupifang (+++).

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